



# The inhibition of N<sub>2</sub>O production by ocean acidification in cold temperate and polar waters



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## ABSTRACT

The effects of ocean acidification (OA) on nitrous oxide (N<sub>2</sub>O) production and on the community composition of ammonium oxidizing archaea (AOA) were examined in the northern and southern sub-polar and polar Atlantic Ocean. Two research cruises were performed during June 2012 between the North Sea and Arctic Greenland and Barent Seas, and in January–February 2013 to the Antarctic Scotia Sea. Seven stations were occupied in all during which shipboard experimental manipulations of the carbonate chemistry were performed through additions of NaHCO<sub>3</sub> + HCl in order to examine the impact of short-term (48 h for N<sub>2</sub>O and between 96 and 168 h for AOA) exposure to control and elevated conditions of OA. During each experiment, triplicate incubations were performed at ambient conditions and at 3 lowered levels of pH which varied between 0.06 and 0.4 units according to the total scale and which were targeted at CO<sub>2</sub> partial pressures of ~500, 750 and 1000 μatm. The AOA assemblage in both Arctic and Antarctic regions was dominated by two major archetypes that represent the marine AOA clades most often detected in seawater. There were no significant changes in AOA assemblage composition between the beginning and end of the incubation experiments. N<sub>2</sub>O production was sensitive to decreasing pH<sub>T</sub> at all stations and decreased by between 2.4% and 44% with reduced pH<sub>T</sub> values of between 0.06 and 0.4. The reduction in N<sub>2</sub>O yield from nitrification was directly related to a decrease of between 28% and 67% in available NH<sub>3</sub> as a result of the pH driven shift in the NH<sub>3</sub>:NH<sub>4</sub><sup>+</sup> equilibrium. The maximum reduction in N<sub>2</sub>O production at conditions projected for the end of the 21st century was estimated to be 0.82 Tg N y<sup>-1</sup>.

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## 1. Introduction

Anthropogenic activities currently add 10 PgC per year to the atmosphere as CO<sub>2</sub>. The change in atmospheric CO<sub>2</sub> from ~280 ppm (ppm) in pre-industrial times to 395 ppm in 2013 (Le Quéré et al., 2014) has impacted the earth system on several scales. The oceans and atmosphere are intimately linked so that changes to the partial pressure of atmospheric CO<sub>2</sub> result in proportional changes in dissolved CO<sub>2</sub> in the marine environment. As a result of this, the rise of global temperatures due to an enhanced greenhouse effect has been buffered by the exchange of approximately 25% of anthropogenic CO<sub>2</sub> into the oceans (Le Quéré et al., 2014) and it is this condition that has resulted in a profound change to ocean carbonate chemistry and the phenomenon of ocean acidification (OA) (Raven et al., 2005). As a consequence pH has decreased by 0.0011–0.0024 units per year for the last two

decades (Rhein et al., 2013), so that oceanic pH is on average ~0.1 units lower than it was prior to the industrial revolution. This pH decrease equates to an increase in acidity of 26% and further increases are predicted. Projections afforded by the current generation (CMIP5) of model-based scenarios (Moss et al., 2010) indicate decreasing ocean pH of between 0.06 and 0.33 units for the 2090s relative to the 1990s for “high mitigation” RCP2.6 and the “business as usual” RCP8.5 scenarios respectively (Bopp et al., 2013; Ciais et al., 2013). Based on our current understanding of microbial biogeochemistry, it is expected that elevated oceanic pCO<sub>2</sub> and the subsequent decrease in pH will have direct (e.g. the increase in N<sub>2</sub> and CO<sub>2</sub> fixation by *Trichodesmium*; Hutchins et al., 2007) and indirect (e.g. the increase in shallow water remineralisation associated with a reduced ballast effect; Hofmann and Schellnhuber, 2009) impacts on microbial nutrient cycling. It is considered that these effects may fundamentally alter current biogeochemical cycles (e.g. Codispoti, 2010).

N<sub>2</sub>O is a trace gas whose atmospheric concentration is increasing at a mean rate of ~0.75 ppb yr<sup>-1</sup> (Hartmann et al.,

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2013). It is a greenhouse gas with a global warming potential on a 100 year timescale of approximately 300 times that of  $\text{CO}_2$  (Ramaswamy et al., 2001) and it contributes significantly to stratospheric ozone depletion (Ravishankara et al., 2009). Though the  $\text{N}_2\text{O}$  concentration in most of the surface of the global ocean is in close equilibrium with the atmosphere (Nevison et al., 1995), the oceans contribute about 30% of the natural  $\text{N}_2\text{O}$  source to the atmosphere (Bange, 2006) and there is a fine balance between the oceans acting as net producer or consumer of  $\text{N}_2\text{O}$ . Environmental effects associated with a changing climate, which include rising temperatures, oxygen depletion and ocean acidification are quite likely to impact the level of this equilibrium (Codispoti, 2010).

Nitrous oxide is biologically produced through three processes: nitrification involves the two stage aerobic oxidation of  $\text{NH}_4^+$  through  $\text{NO}_2^-$  to  $\text{NO}_3^-$ , where the release of  $\text{N}_2\text{O}$  as a by-product is dependent on the ambient  $\text{O}_2$  concentration (Goreau et al., 1980; Loescher et al., 2012). Denitrification is the anaerobic transformation of  $\text{NO}_3^-$  into  $\text{N}_2$  which has  $\text{N}_2\text{O}$  as an intermediate. In the third route, nitrifier-denitrification,  $\text{N}_2\text{O}$  can be formed during the reduction of  $\text{NO}_2^-$  via nitric oxide to  $\text{N}_2\text{O}$ . In the open oceans nitrification is the dominant mechanism for the production of  $\text{N}_2\text{O}$  (Freing et al., 2012), and in the limited number of studies that have been reported, this process has been shown to be particularly sensitive to OA in sub-surface marine waters. Huesemann et al. (2002) found a linear reduction in nitrification rate with high additions of  $\text{CO}_2$ , so that at pH 6.5, nitrification was reduced by up to 90% of the natural condition. That experiment was more relevant to the effects associated with  $\text{CO}_2$  disposal than with levels of OA predicted for the coming century. In a study where pH was manipulated between 8.09 and 7.42 at several locations in the Pacific and Atlantic, Beman et al. (2011) showed unequivocal evidence for an inhibitory effect of short term OA on nitrification. Kitidis et al. (2011) manipulated conditions of pH between ambient and 6.5 for water column samples from the English Channel and found that ammonium oxidation rate decreased with decreasing pH to near complete inhibition at pH 6.5.

It has been hypothesized that nitrification rates may be altered by increasing OA either directly, by impacting on microbial physiology or community composition, or indirectly by changes to the supply of organic material (Codispoti, 2010). Hutchins et al. (2009) speculated that increasing levels of  $\text{CO}_2$  may lead to an increase in autotrophic nitrification rates through a reduction of  $\text{CO}_2$  limitation or  $\text{CO}_2$  fertilization effect. To date this effect has not been observed for the open ocean and the limited number of studies have shown some equivocality in their findings. Clark et al. (2014) performed an investigation of OA on nitrification and  $\text{N}_2\text{O}$  in near surface ( $\sim 5$  m) waters of the NW European shelf seas. The absence of any relationship between OA and  $\text{N}_2\text{O}$  observed is, in part, attributed to the oxygenated status of these waters and the low production of  $\text{N}_2\text{O}$  expected. Clark et al. also observed variability in the impact of OA on nitrification which did not allow a relationship or mechanistic understanding of the relationship between OA and nitrification to be developed. In a study of estuarine and near coastal waters of Narragansett Bay, Fulweiler et al. (2011) found that nitrification rates increased along decreasing natural gradients of pH. Whilst not dismissing the  $\text{CO}_2$  fertilisation effect, Fulweiler et al. concluded that a combination of environmental conditions was likely to be the biggest driver influencing nitrification. The decrease in rates could be associated with change in the microbial community possibly as a result of competitive strength to compete for available  $\text{NH}_4^+/\text{NH}_3$ , or as a result of reduced  $\text{NH}_3$ , the favored substrate for the first stage of nitrification (Ward, 2008a). The  $\text{NH}_4^+:\text{NH}_3$  equilibrium has a  $pK_a$  of  $\sim 9.2$ , favouring  $\text{NH}_4^+$  in an acidifying ocean.

Changes in the microbial ammonium oxidising community composition as a result of changing OA have been reported by

Bowen et al. (2013) who saw greater changes in assemblage composition and abundance of ammonium oxidising bacteria (AOB) than of ammonium oxidising archaea (AOA). Whilst both AOB and AOA are found throughout the oceans and produce  $\text{N}_2\text{O}$  (Santoro et al., 2011; Loescher et al., 2012) AOA are considered to be the principal nitrifying organisms (Wuchter et al., 2006).

To our knowledge, there are no reports to date which confirm the inhibition of  $\text{N}_2\text{O}$  production by OA, although the limited evidence from studies on nitrification indicate that this is to be expected. Decreasing oceanic oxygen and decreasing ballast effect (sinking rate of particles) are both predicted to increase the release of  $\text{N}_2\text{O}$  from nitrification in a future ocean (Codispoti, 2010; Gehlen et al., 2011) and so prediction of the overall  $\text{N}_2\text{O}$  inventory and net emissions will prove problematic. Beman et al. (2011) performed an indicative budget for  $\text{N}_2\text{O}$  production from nitrification, which suggested that a decrease in nitrification between 3% and 44% (the range indicated by Beman for the next 20 to 30 years) could result in reduced  $\text{N}_2\text{O}$  emissions comparable to all current  $\text{N}_2\text{O}$  production from fossil fuel combustion and industrial processes.

Here we report on two studies (Tyrrell et al., 2016) performed to investigate the impact of OA on  $\text{N}_2\text{O}$  production and AOA distribution as a contribution to the United Kingdom Ocean Acidification (UKOA) program. Experiments were performed at ocean stations (E01–E05) during cruise JR271 of the RRS James Clark Ross between the North Sea and Arctic Ocean in June 2012, and at stations (B03 and B04) during cruise JR274 to the Southern Ocean in January–February 2013 (Fig. 1).

## 2. Methods

Seawater was collected from Niskin bottles deployed on a titanium frame at the base of the surface mixed-layer defined by the temperature profile (except for E04). The Niskin bottles were transferred to a positive pressure Class-100 filtered trace metal clean container to avoid contamination. Unfiltered water was dispensed into 4.5 L polycarbonate incubation bottles and the bottles were closed pending carbonate chemistry manipulation. Single experimental bottles were individually manipulated to achieve a range of 4 different target  $p\text{CO}_2$  levels (ambient, 550, 750 and 1000  $\mu\text{atm}$ ), according to the initial carbonate chemistry of the seawater at the time of the water collection. The manipulation of the carbonate system was achieved through additions of  $\text{NaHCO}_3^- + \text{HCl}$  (Borowitzka, 1981; Gattuso and Lavigne, 2009; Schulz et al., 2009) and immediately verified by total alkalinity (TA) and DIC analyses. Values of  $p\text{CO}_2$  and  $\text{pH}_T$  (total scale) were determined using CO2sys (Lewis and Wallace, 1998). Following manipulation of  $p\text{CO}_2$ , bottles were closed with PTFE backed butyl septa before being further sealed with parafilm and incubated.

Tests have been performed previously to confirm the validity of this approach and to test the integrity of the polycarbonate bottles to  $\text{N}_2\text{O}$  diffusion. At six positions on a transect through the Atlantic Ocean between the UK and the Falkland Islands, replicate bottles (2–4) were filled with seawater and poisoned with the addition of 1 ml saturated  $\text{HgCl}_2$  and incubated in the dark at collection temperature.  $\text{N}_2\text{O}$  concentration was determined on collection, and thereafter triplicate analyses of  $\text{N}_2\text{O}$  were made at several time points over 6 days.  $\text{N}_2\text{O}$  concentration remained stable during each of these storage tests, the coefficient of variation varied between 1.3% and 5.2% (mean 3.9%,  $n=16$  time points  $\times 3$  analyses).  $F$  tests between  $\text{N}_2\text{O}$  in killed samples and initial concentrations, and between killed samples and the expected (atmospheric equilibrium) concentration confirmed 86% and 87% similarity in variance respectively.

During this study,  $\text{N}_2\text{O}$  concentration was determined in triplicate on samples first prepared (T0) and after 48 h (T48) of

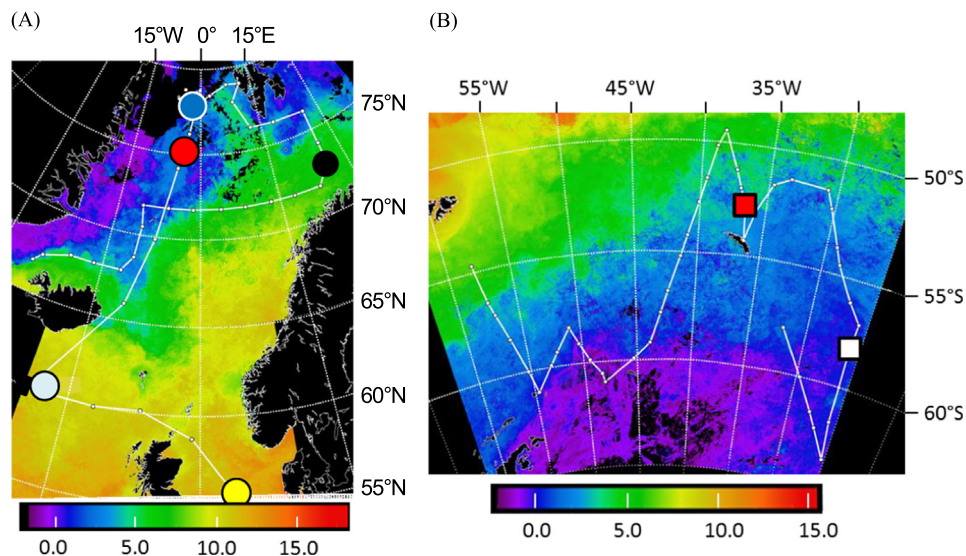


Fig. 1. Location of experiments performed during (a) JR271 in June 2012 and (b) JR274 in January–February 2013.

incubation. Subsamples from the incubations were filtered for determination of AOA at T0 and at several time points afterwards. Incubations were performed in the dark within a purpose-built experimental laboratory container allowing precise temperature control. The temperature was adjusted to the in situ value at the time of the water collection. Temperature within a dummy incubation bottle was monitored using a traceable thermometer, while two recording thermometers were used to monitor air temperature in the incubator. Samples were also collected for N<sub>2</sub>O analysis from CTD casts performed at each of the experimental stations during JR271 only.

### 2.1. N<sub>2</sub>O analysis

Samples were collected using acid cleaned Tygon tubing directly from CTD Niskin bottles or by siphoning from 4.5 L incubation bottles into 1 L borosilicate flasks. Single samples were taken from CTD bottles and triplicates from the incubated sample. Samples were overfilled in order to expel air bubbles, poisoned with 200  $\mu$ L of saturated HgCl<sub>2</sub> solution and temperature equilibrated at  $25.0 \pm 0.5$  °C. In all cases samples were analyzed within 8 h of collection. N<sub>2</sub>O was determined by single-phase equilibration gas chromatography with electron capture detection similar to that described by Upstill-Goddard et al. (1996). Each individual sample was calibrated against three certified ( $\pm 5\%$ ) reference standards of 287, 402 and 511 ppb (Air Products Ltd.) which are traceable to NOAA WMO-N2O-X2006A scale for N<sub>2</sub>O mole fractions. Mean instrument precision from daily, triplicate analyses of the three calibration standards ( $n=81$ ) was 0.95%. Concentrations of N<sub>2</sub>O in seawater were calculated from solubility tables of (Weiss and Price, 1980) at equilibration temperature ( $\sim 25$  °C) and salinity. All N<sub>2</sub>O data from this study are available from the British Oceanographic Data Centre according to. <http://dx.doi.org/10.5285/268dfd3b-dcc6-3f4a-e053-6c86abc0c2f9>.

### 2.2. amoA AOA microarray

The array (BC016) was developed following the archetype array approach described and employed previously (e.g. Ward and Bouskill, 2011; Bowen et al., 2013), with 90-mer oligonucleotide probes. Each probe included an amoA-specific 70-mer region and a 20-mer control region (5'-GTACTACTAGCCTAGGCTAG-3') bound to a glass slide. The design and spotting of the probes have been

described previously (Bulow et al., 2008). Using an established algorithm (Bulow et al., 2008), 99 different archetypes were identified representing 8296 archaeal amoA sequences, which had been aligned and analyzed by Biller et al. (2012). The Archetype probes are numbered according to their representation in the database; of the > 8000 sequences aligned by Biller et al. (2012), the largest number of them, 1191 are represented by AOA1, 1149 by AOA2 and 795 by AOA3, and so forth. The probe accession numbers and sequences are listed in Table S1.

### 2.3. Target preparation, microarray hybridization, and data analysis

Seawater samples (up to 8 L) were filtered onto 0.2  $\mu$ m pore size Sterivex filters (Millipore, Billerica, MA) using a peristaltic pump, and filters were flash frozen in liquid nitrogen and stored at  $-80$  °C. Total DNA and RNA were extracted from Sterivex filters using the AllPrep DNA/RNA Kit (Qiagen Sciences) with slight modifications (as in Ward, 2008b) to the manufacturer's instructions. The extraction procedures were performed twice on each Sterivex filter in order to maximize the DNA yield. cDNA was made from the RNA immediately upon purification using the Superscript III Kit (Life Technologies) following the manufacturer's instructions after concentrating the RNA extract using MinElute columns (Qiagen Sciences).

Archaeal amoA genes were amplified from the DNA and cDNA using primers Arch-amoAF (5'-STAATGGTCTGGCTTAGACG-3') and Arch-amoAR (5'-GCGGCCATCCATCTGTATGT-3') and protocols as described by Francis et al. (2005). DNA and cDNA from To and Tf (Tf=T final=96 h for all five Arctic experiments, 144 h for Antarctic experiment B03 and 168 h for Antarctic experiment B04). Targets for microarray hybridization were prepared from the amoA PCR products according to Ward and Bouskill (2011), hybridized in duplicate on a microarray slide and washed as described previously (Ward and Bouskill, 2011). Washed slides were scanned using a laser scanner 4300 (Agilent Technologies, Palo Alto, CA) and analyzed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA). Quantification of hybridization signals was performed as described previously (Ward and Bouskill, 2011). A normalized fluorescence ratio (FRn) for each archetype was calculated by dividing the fluorescence signal of the archetype by the highest fluorescence signal within the same array. Then the FRn of each archetype from the duplicate arrays were averaged. The relative fluorescence ratio (RFR) of each archetype was calculated as the



contribution of FRn of the archetype to the sum of FRn of all *amoA* archetypes on the array and averaged for duplicate arrays from each sample. The original array data are available at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo/>) at the National Center for Biotechnology Information under GEO Accession no. GSE72072.

#### 2.4. Statistical analysis

The array data were analyzed using the vegan package in R (CRAN website; <http://www.R-project.org/>) (Borcard et al., 2011). RFR values were transformed (Arcsin(Square root)) in order to normalize the proportional data. Environmental data were transformed (square root) and then standardized around zero (deco-standard in vegan). The transformed data were used in all diversity and ordination analyses according to Borcard et al. (2011). The null hypothesis that the *amoA* community composition did not differ between groups of samples (e.g., time points or experiments) was tested using Multi-response Permutation Procedure (MRPP) (Zimmerman et al., 1985) with a significance level of 5%.

### 3. Results

#### 3.1. Station environmental characteristic

A full description of the carbonate chemistry during both cruises is presented by Tynan et al. (2016). During cruise JR271 in the Arctic region, the range of conditions experienced ranged between the nutrient poor northern North Sea to higher surface nitrate concentrations within the sea-ice of the Fram Strait in the Greenland Sea (Table 1). Experimental stations E01 and E02 were representative of the summertime North Atlantic and can be seen to cluster closely with respect to their surface temperature and salinity signal (Fig. 2), but are distinct with regards  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and  $\text{pCO}_2/\text{pH}$  signals (Table 1). Temperature at the depth of sample collection during JR271 (Fig. 3) ranged between  $-1.76^\circ\text{C}$  at station E04 and  $9.49^\circ\text{C}$  at station E02. For the 5 stations occupied during JR271,  $\text{pCO}_2$  was maximal at E02 at 391.5 (pH minimum of 8.051) and decreased to a minimum of 263.0  $\mu\text{atm}$  at E04 (maximum pH of 8.186) within the Arctic ice-fields.

Southern Ocean stations B03 and B04 during JR274 were selected primarily for the benefit of other investigations to have contrasting conditions of phytoplankton biomass, with low chlorophyll concentrations of  $0.63 \mu\text{g L}^{-1}$  at B03 and higher levels of  $4.19 \mu\text{g L}^{-1}$  at E04. Temperatures at the depth of sample collection were  $0.89$  and  $-1.20^\circ\text{C}$  at B03 and B04 respectively (Fig. 4). The highest ambient  $\text{pCO}_2$  and lowest pH levels out of all seven stations were experienced at B03 at 425.6  $\mu\text{atm}$  and 7.999 respectively. Experimental sample collection was performed as soon as possible after arrival at the station position and before

water column profiles of  $\text{N}_2\text{O}$  could be performed. Seawater for experimental manipulation was collected from the base of the surface mixed layer, which was determined from the temperature profile, to coincide with expected maxima in near surface  $\text{N}_2\text{O}$  concentration and production from nitrification (e.g. Forster et al., 2009; Kitidis et al., 2010). During JR271 this approach proved successful at stations E01, E02, and E05 (Fig. 3a, b and e) where  $\text{N}_2\text{O}$  concentrations were elevated relative to those at the immediate surface indicative of active production at these depths. At stations E03 and E04, freshwater associated with melting ice in the near surface created a stronger defined pycnocline at approximately 38 m and 18 m respectively (inset on Fig. 3c and d) which appeared to favor nitrification and  $\text{N}_2\text{O}$  production and meant that sampling at 60 m and 40 m was of waters with reduced  $\text{N}_2\text{O}$  and apparently lower production rates.

Water column profiles of dissolved  $\text{N}_2\text{O}$  were not determined during JR274.

#### 3.2. OA impact on $\text{N}_2\text{O}$ concentration

Over the 48 h period of incubation  $\text{N}_2\text{O}$  was produced in all bottles which had received no amendment to their carbonate chemistry. That is, there was a mean production of  $\text{N}_2\text{O}$  of 5.9, 6.6, 1.4, 0.3 and 3.6  $\text{nmol L}^{-1}$  in two days for stations E01–E05 respectively during JR271 and of 2.2 and 1.1  $\text{nmol L}^{-1}$  for stations B03 and B04 respectively during JR274. In all cases the concentration of  $\text{N}_2\text{O}$  decreased linearly with increasing OA (Fig. 4, Table 2) between ambient pH and pH 7.75. The observed decrease in  $\text{N}_2\text{O}$  concentration between treatments ranged between 12.4% and 43.3% of the ambient condition during JR271. The largest changes in response to OA treatment were experienced at stations E01, E02 and E05 (37.7%, 43.3% and 23.8% respectively) where water sample collection was closest to the region of maximum

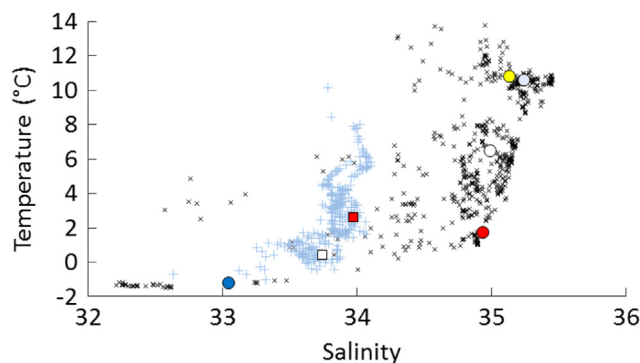
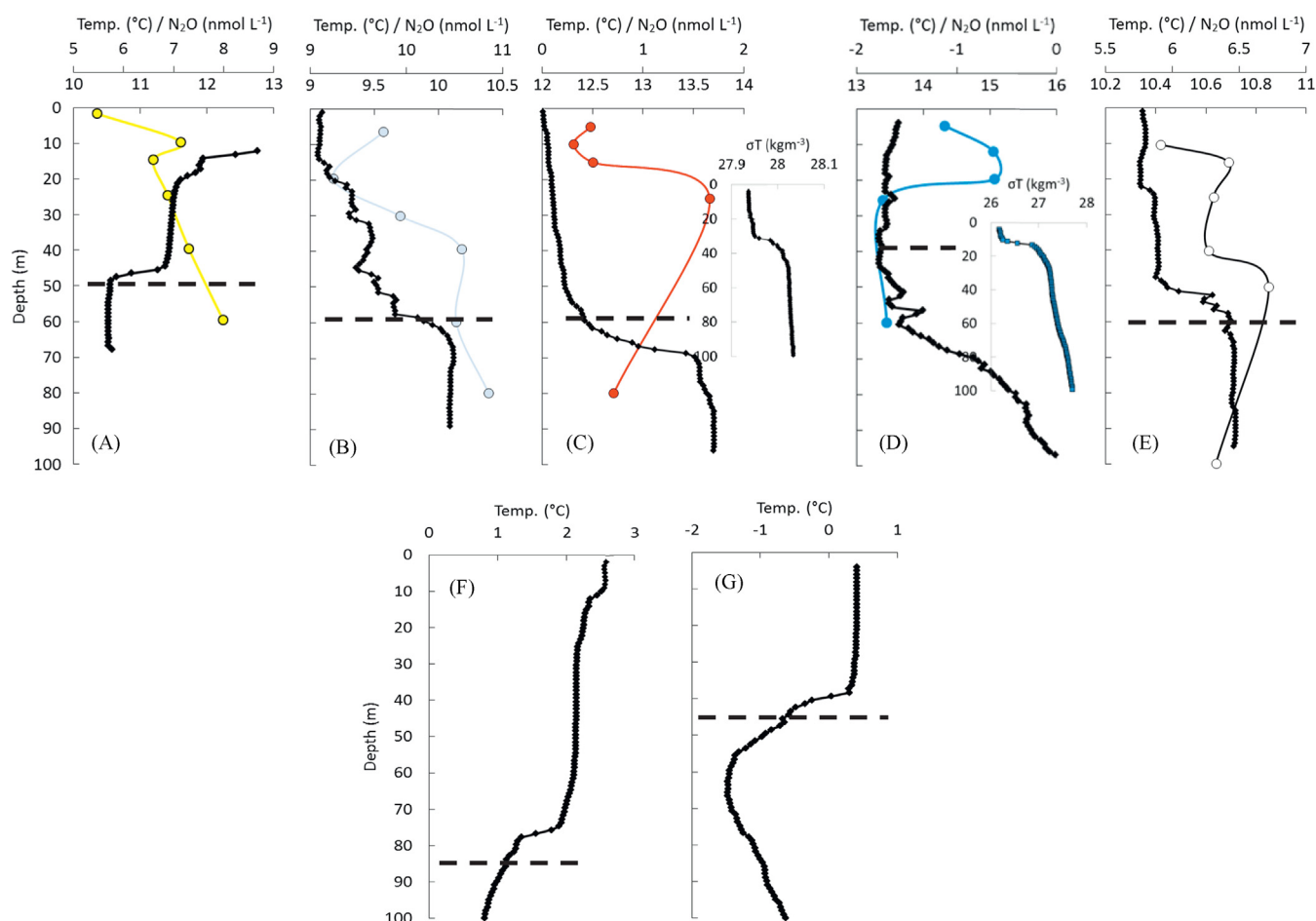


Fig. 2. Surface temperature versus salinity for cruises JR271 (X) and JR274 (+). Experimental stations are represented by E01 (●), E02 (○), E03 (●), E04 (●), E05 (●), B03 (■) and B04 (□).

Table 1

Location of ocean acidification experiments and environmental variables encountered during JR271 (June 2012) and JR274 (January–February 2013).

Experiment	Latitude	Longitude	Depth (m)	Temp ( $^\circ\text{C}$ )	$\text{NO}_3^-$ ( $\mu\text{M}$ )	$\text{NH}_4^+$ (nM)	pH	$\text{pCO}_2$ ( $\mu\text{atm}$ )	Location
<b>JR271</b>									
E01	56.267°N	02.633°E	50	6.7	0.65	44	8.105	339.4	North Sea
E02	60.594°N	18.857°W	60	9.49	9.18	452	8.051	391.5	South of Iceland
E03	76.175°N	02.549°W	60	0.16	10.1	502	8.152	292.0	Greenland Sea
E04	78.353°N	03.664°W	40	$-1.76$	4.16	69	8.186	263.0	1. Ice-Edge Greenland Sea
E05	72.892°N	26.001°E	60	6.01	8.76	178	8.110	331.4	1. In-Ice Barents Sea
<b>JR274</b>									
B03	52.69°S	36.63°W	85	0.89	25.83		7.999	435.6	North of South Georgia
B04	58.08°S	25.93°W	46	$-1.2$	21.51		8.093	343.4	East of S. Sandwich Islands



**Fig. 3.** Temperature and N<sub>2</sub>O profiles for each of the experimental stations (a) E01, (b) E02, (c) E03, (d) E04, (e) E05, (f) B03, and (g) B04. In panels (c) and (d) the inset panel shows the density profile ( $\sigma T$ ). In all cases the depth of seawater sample collection is indicated by the dashed line. N<sub>2</sub>O profiles were not performed during B03 and B04.

N<sub>2</sub>O concentration (Fig. 3), which is assumed to be coincident with maximum production. At E03 and E04, sample water collection was approximately 35 m and 20 m below the depths of maximum N<sub>2</sub>O, and changes in N<sub>2</sub>O concentration with decreasing pH are less apparent (16.1% and 12.4% respectively), though still significantly correlated ( $p < 0.02$ ). During JR274, water column profiles of N<sub>2</sub>O were not made and so it is not possible to relate the properties of the incubated samples to the natural distribution of N<sub>2</sub>O. Decreases in N<sub>2</sub>O with increasing OA were observed (Fig. 4, Table 2) though these were low relative to observations during JR271 (13.1% and 4.0% for B03 and B04 respectively).

#### 4. AOA assemblage

In the samples from the Arctic cruise JR271, 42 of the 99 archetypes were detected at 1% of the total hybridization signal in the five experiments. MRPP analysis showed that there were no significant differences in assembly composition for comparisons among stations, between To versus Tf, or between DNA versus RNA for ambient or  $\sim 1000 \mu\text{atm}$ . The RFR values for all samples were averaged to evaluate the relative abundance of the archetypes. Of the 42 archetypes detected at  $> 1\%$  of the signal, only three had an average RFR greater than 5%. The overwhelmingly strongest signal was due to Archetype AOA3, which represents sequences from the marine water column. The second strongest signal was from Archetype AOA1, which also represents marine water column and sediment sequences, and includes the cultivated ammonia-oxidizing archaeon *Nitrosopumilus maritimus*. The third and

fourth largest RFRs were Archetypes AOA73 and AOA83, which represented only five and two sequences, respectively, in the Biller et al. (2012) database, all from sediments or soils. The RFR for the five archetypes that had the highest average signals across all samples are shown in Supplementary Fig. 1.

Consistent with the MRPP analysis, PCA clustered most of the samples together (Fig. 5). Three samples from E01, however, were distinct. DNA from 1TfAmbD and RNA from 1ToAmbR to 1Tf1000R had the greatest Archetype richness ( $N$ , number of Archetypes detected at  $> 1\%$  of total signal) at 24, 17 and 15 Archetypes respectively. The next richest sample was 2Tf1000D with 13 significant Archetypes. In the other 27 samples,  $N$  ranged from 4 to 10.

Twenty-four of the 99 Archetypes were detected at 1% of the total hybridization signal in the two Antarctic experiments from JR274. The top three Archetypes were AOA3, AOA1 and AOA73 as above, but only AOA3 and AOA1 had an average RFR greater than 5%. The fourth strongest signal was AOA7, a marine water column clade representing 296 sequences in the Biller et al. (2012) database. The RFR for the five archetypes that had the highest average signals across all samples are shown in Supplementary Fig. 2.

PCA clustered the assemblages by station and between RNA and DNA (Fig. 6). MMRP analysis indicated that the assemblages in Experiments 3 and 4 were significantly different ( $p < 0.004$ ) and that the composition of the assemblages represented in the DNA was significantly different from that in the RNA ( $p < 0.013$ ). These differences are due to different contributions of the smaller signals; AOA1 and AOA3 were the strongest signals in all DNA and RNA samples. There were no significant differences between To

and Tf in DNA or RNA for the two experiments ( $p < 0.603$ ). Archetype richness was greater in Experiment B04 (average  $N=10.3$ ) than in Experiment B03 (average  $N=7$ ) and the overall range was 4–15.

5. Discussion

The AOA assemblage in both Arctic and Antarctic regions was dominated by two major archetypes that represent the marine AOA clades most often detected in seawater. Although several other archetypes were present, the dominance of AOA1 and AOA3 implies that their physiological characteristics control the rates of

nitrification and their sensitivity to OA. The recently described oceanic AOA isolate (Santoro et al., 2015), *Candidatus Nitrosopelagicus brevis*, is a member of the AOA3 archetype, suggesting it is characteristic of the most abundant AOA in the polar oceans.

There were no significant changes in AOA assemblage composition between the beginning and end of the incubation experiments, probably because all the samples were overwhelmingly dominated by the two most abundant archetypes. The incubation period was not long enough to cause significant turnover in the assemblage, even if OA did exert differential selective pressure on the assemblage members. Significant differences were found between Antarctic stations B03 and B04, and between RNA and DNA at these stations, but not among treatments. The observed differences were due to varying relative contributions of the lower abundance archetypes and therefore imply little about the differential activity or response of the assemblage as a whole, although differential effects on  $N_2O$  production among the lower abundance archetypes cannot be ruled out. This apparent lack of sensitivity of the AOA assemblage to short term OA is consistent with the previous observation of Bowen et al. (2013) that AOA assemblages are not very sensitive to small pH changes in both long (3 weeks) and short (6 days) exposures.

Sample collection for the OA manipulation experiments was directed towards the base of the euphotic zone, and samples were

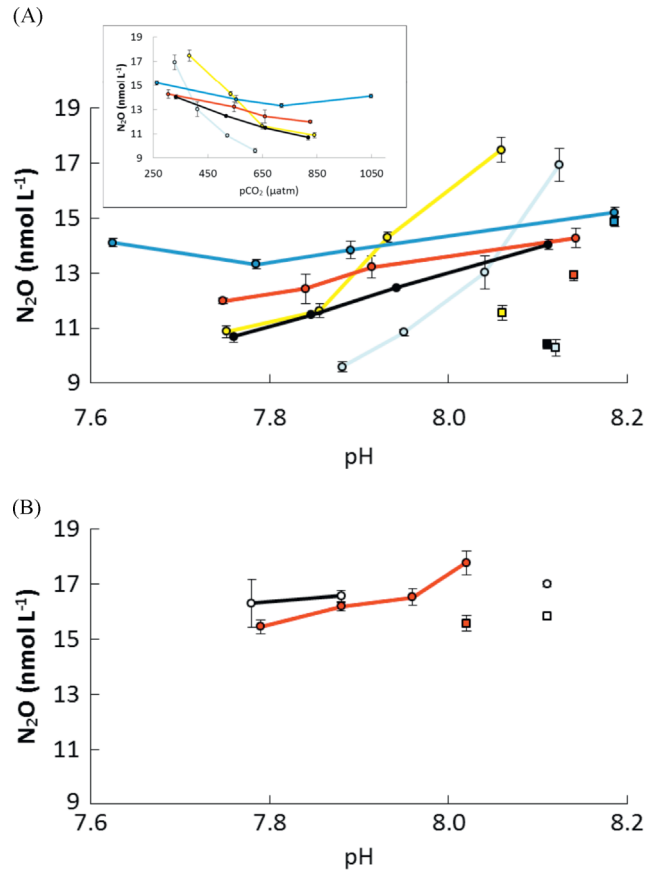


Fig. 4. Mean  $N_2O$  concentration ( $\pm 1$  sd) against  $pH_T$  during  $CO_2$  manipulation experiments during (a) JR271, with the inset showing the same  $N_2O$  data against  $pCO_2$ , and (b) JR274. In both panels, squares represent the ambient concentration at T0, circles are  $N_2O$  concentration after 48 h incubation. In (a) E01 yellow, E02 turquoise, E03 red, E04 blue, E05 black. In panel (b) B03 red, B04 white.

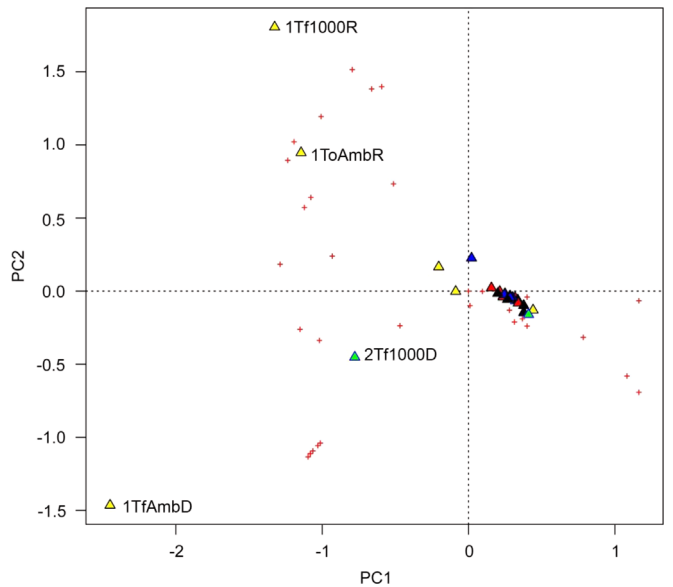
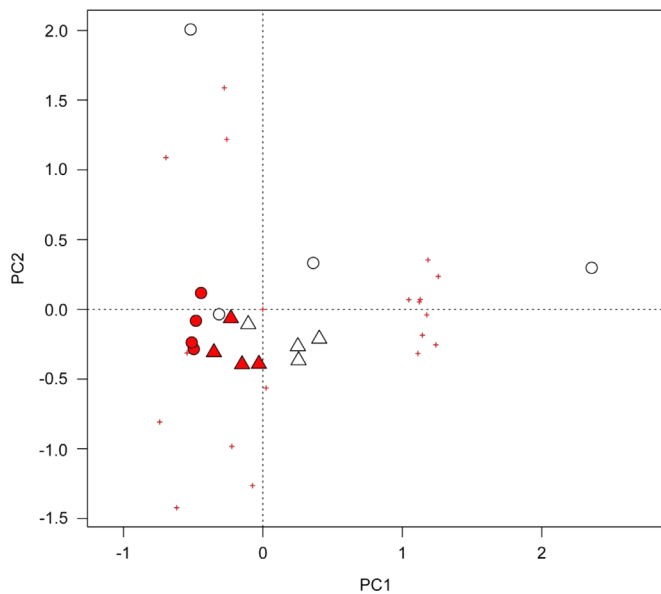


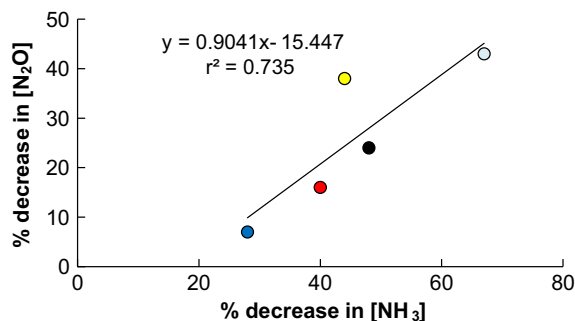
Fig. 5. PCA of AOA during JR271. Stations are color coded, E01=yellow, E02=turquoise, E03=red, E04=blue, E05=black. R=RNA (triangles); D=DNA (circles). To=initial; Tf=final. Amb=ambient; 1000=highest  $CO_2$  treatment. Red crosses represent individual AOA archetypes.

Table 2  
The linear relationship between  $N_2O$  and pH for the 7 stations occupied during JR271 and JR274.

Experiment	Ambient pH	Manipulated pH range	Regression	$r^2$
JR271				
E01	8.105	8.06–7.75	$N_2O=22.44 \text{ pH} - 163.7$	0.947
E02	8.051	8.12–7.88	$N_2O=29.721 \text{ pH} - 225.15$	0.956
E03	8.152	8.14–7.75	$N_2O=5.8887 \text{ pH} - 33.616$	0.977
E04	8.186	8.19–7.63	$N_2O=2.4324 \text{ pH} - 5.0328$	0.524
		8.19–7.79	$N_2O=4.6811 \text{ pH} - 23.114$	0.999
E05	8.110	8.11–7.79	$N_2O=9.5179 \text{ pH} - 63.166$	0.999
JR274				
B03	8.02	8.02–7.79	$N_2O=9.2705 \text{ pH} - 56.879$	0.907
B04	8.11	8.11–7.78	$N_2O=2.0183 \text{ pH} + 0.6177$	0.987



**Fig. 6.** PCA of AOA during JR274. Stations are color coded, B03=red, B04=white. DNA=circles; RNA=triangles. Red crosses represent individual AOA archetypes.



**Fig. 7.** Mean percentage decrease in observed  $N_2O$  concentration associated with estimated decreases in  $NH_3$  concentration during JR271. E01 yellow, E02 turquoise, E03 red, E04 blue, E05 black.

incubated in the dark and so it is considered that competition from phytoplankton for available  $NH_3$  or  $NH_4^+$  is likely to be low. As the nitrifying AOA community composition proved resilient to changes in OA it would seem that observed changes in  $N_2O$  concentration were more likely associated to an altered  $NH_4^+ : NH_3$  equilibrium (Hutchins et al. 2009; Beman et al., 2011). Beman et al. (2011) suggested that the decline in  $NH_3$  with increasing OA is the driving factor in the reduced rates of  $NH_4^+$  oxidation observed during their study.  $NH_3$  is the substrate used in the first stage of nitrification (Ward, 2008a) and the equilibrium equations of Bell et al. (2007, 2008) indicate that at constant temperature and salinity,  $NH_3$  concentrations in seawater would decrease by 50% over a pH change from 8.1 to 7.8 (Wyatt et al., 2010). Using the equations presented by Bell et al. we estimated concentrations of  $NH_3$  from calculated  $pK_a$  and in-situ determined  $pH_T$  and  $NH_4^+$  concentration. Using a fixed value of  $pK_a$  of 9.3, Beman et al. (2011) estimated that  $NH_3$  typically represented 6.3% of total  $NH_x$ . At the stations sampled during JR271, using  $pK_a$  determined from ambient temperature and salinity (9.9, 9.9, 10.1, 10.2, 10.0 for stations E01 to E05 respectively), we estimate a mean contribution of  $NH_3$  to  $NH_x$  of  $0.68 \pm 0.15\%$ . For the manipulated conditions of pH during JR271 (Table 2) the  $NH_3$  concentration is estimated to decrease to between 28% and 67% of the ambient concentration at

the start of each experiment. Over wide geographic areas and conditions of pH or  $CO_2$  manipulation Beman et al. (2011) found significant correlations between percentage changes in  $NH_3$ , pH and ammonium oxidation rates, as did Huesemann et al. (2002) and Kitidis et al. (2011) between pH and ammonium oxidation. During the current study we have shown similar relationships between absolute values of pH and  $N_2O$  (Table 2) and in Fig. 7 between the percentage decrease in  $N_2O$  during incubation experiments and the decrease in  $NH_3$  ( $r^2=0.735$ ,  $p<0.1$ ) as a result of the induced shift in the  $NH_4^+ : NH_3$  ratio. Our results indicate that changes in  $pH_T$  of between 0.23 and 0.4 (mean=0.31) for these ocean regions, which range between northern temperate and Arctic and Antarctic sea-ice, are likely to result in decreases of dissolved  $N_2O$  of between 12% and 43% (mean=21%), as a result of a reduced  $NH_3$  regime of between 28% and 67%. These changes in  $pH_T$  are towards the higher end of those conditions projected for the late 21st century by CMIP5 simulations, e.g. Bopp et al. (2013) indicate decreases of 0.22 and 0.33 pH units for RCP6.0 and RCP8.5 respectively, whilst Gattuso et al. (2015) indicate a range of decrease of 0.14–0.4 pH units for RCP2.6 and RCP8.5 respectively. Both Beman et al. (2011) and Kitidis et al. (2011) observed a decline in ammonium oxidation rates even at their lowest manipulated conditions of OA. Our data confirm this sensitivity with decreases in dissolved  $N_2O$  following treatments of between 2.3% and 23% (mean=12%) at the lowest OA manipulations which varied between 0.06 and 0.23 (mean=0.13) pH units.

The decreased production of  $N_2O$  with increasing OA has the potential to offer a negative feedback to a warming environment by reducing the atmospheric radiative forcing contribution of  $N_2O$ . This could derive from two mechanisms: a direct reduction in the flux of  $N_2O$  from the ocean to the atmosphere, which could be further exacerbated by a reversal of the direction of flux, should the ocean change from source to sink of atmospheric  $N_2O$ . Beman et al. (2011) estimated that their observations of decreased nitrification rates (3–44%), would lead to a global decrease in  $N_2O$  production of between 0.06 and 0.83  $Tg\ N\ y^{-1}$  in the next 20 to 30 years. This is of particular note as it is comparable to all current  $N_2O$  production from fossil fuel combustion and industrial processes (0.7  $Tg\ N\ y^{-1}$ ). By taking a similar approach and assuming that 50% of the global ocean source of  $N_2O$  of 3.8  $Tg\ N\ y^{-1}$  (Denman et al., 2007) is produced through nitrification (Codispoti, 2010), the data from the current study indicate comparable, albeit slightly lower reductions in oceanic  $N_2O$  production. For the lower range of treatments (mean  $pH_T$  decrease=0.13) the estimated reduction in the ocean  $N_2O$  source is between 0.04 and 0.44  $Tg\ N\ y^{-1}$  and for the highest treatments (mean  $pH_T$  decrease=0.31) the predicted decrease ranges between 0.23 and 0.82  $Tg\ N\ y^{-1}$ .

Our experiments have shown that OA will decrease the production of  $N_2O$  in the pelagic water column. It is though clearly apparent that our future oceans will not undergo OA in isolation from other predicted changes. Warming of the oceans, decreasing oxygen levels (Gruber, 2011; Riebesell and Gattuso, 2015) and an OA induced reduction in the export of organic material to the deep ocean (reduced ballast effect – Codispoti, 2010; Gehlen et al., 2011) are all expected to impact on  $N_2O$  production and release to the atmosphere. Each of these conditions offers a positive feedback to a warming environment with regards their impacts on  $N_2O$  and so to a greater or lesser extent will counter the reduction in  $N_2O$  caused by OA. The individual stressors are identified but combined effects may prove to be additive, synergistic or antagonistic (Riebesell and Gattuso, 2015) and the ultimate impact of these multiple stressors in the ocean offers an unknown, uncharacterised and currently unpredictable control over the release of  $N_2O$  to the atmosphere.



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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.dsr2.2015.12.006>.

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